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ELF4 is a phytochrome-regulated component of a negative-feedback loop involving the central oscillator components CCA1 and LHY

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Summary

Evidence has been presented that a negative transcriptional feedback loop formed by the genes *CIRCADIAN CLOCK ASSOCIATED* (*CCA1*), *LATE ELONGATED HYPOCOTYL* (*LHY*) and *TIMING OF CAB* (*TOC1*) constitutes the core of the central oscillator of the circadian clock in Arabidopsis. Here we show that these genes are expressed at constant, basal levels in dark-grown seedlings. Transfer to constant red light (Rc) rapidly induces a biphasic pattern of *CCA1* and *LHY* expression, and a reciprocal *TOC1* expression pattern over the first 24 h, consistent with initial induction of this synchronous oscillation by the light signal. We have used this assay with wild-type and mutant seedlings to examine the role of these oscillator components, and to determine the function of *ELF3* and *ELF4* in their light-regulated expression. The data show that whereas TOC1 is necessary for light-induced *CCA1/LHY* expression, the combined absence of CCA1 and LHY has little effect on the pattern of light-induced *TOC1* expression, indicating that the negative regulatory arm of the proposed oscillator is not fully functional during initial seedling de-etiolation. By contrast, ELF4 is necessary for light-induced expression of both *CCA1* and *LHY*, and conversely, CCA1 and LHY act negatively on light-induced *ELF4* expression. Together with the observation that the temporal light-induced expression profile of *ELF4* is counter-phased to that of *CCA1* and *LHY* and parallels that of *TOC1*, these data are consistent with a previously unrecognized negative-feedback loop formed by *CCA1/LHY* and *ELF4* in a manner analogous to the proposed *CCA1/LHY/TOC1* oscillator. ELF3 is also necessary for light-induced *CCA1/LHY* expression, but it is neither light-induced nor clock-regulated during de-etiolation. Taken together, the data suggest (a) that ELF3, ELF4, and TOC1 all function in the primary, phytochrome-mediated light-input pathway to the circadian oscillator in Arabidopsis; and (b) that this oscillator consists of two or more interlocking transcriptional feedback loops that may be differentially operative during initial light induction and under steady-state circadian conditions in entrained green plants.

Keywords: ELF4, circadian clock, light input, phytochrome.

Introduction

The Arabidopsis circadian clock generates endogenous rhythms that allow the plant to anticipate daily changes in the light environment by oscillating with a 24 h period that mimics that of the Earth's rotation. In its most simplistic representation, the circadian clock is said to consist of an input pathway, a central oscillator(s), and an output pathway (reviewed by Eriksson and Millar, 2003). Much effort has been devoted to defining the components of the central oscillator, which is thought to consist of an autoregulatory negative-feedback loop(s). To identify oscillator components, altered expression of circadian output genes (genes whose rhythmic expression is controlled by the central clock) has been used extensively in genetic screens. One such screen took advantage of a *CAB2::LUC* line in which the *LUCIFERASE* gene is under the control of the *CAB2* promoter. This line allowed the authors to measure the expression of *CAB2* by luciferase bioluminescence, thereby giving them tools to perform a highly sensitive screen that yielded

several *timing of cab* (*toc*) mutants – mutants showing either long or short periods of *CAB2* oscillations (Millar *et al.*, 1995). One such mutant with a shortened period, *toc1*, has been extensively studied, and the TOC1 protein is postulated to be a component of the Arabidopsis central oscillator (Alabadi *et al.*, 2001; Somers *et al.*, 1998b). *TOC1* was cloned and shown to encode a pseudo-response regulator whose mRNA oscillates in a circadian manner with a peak of expression every evening (Strayer *et al.*, 2000).

Two other important circadian genes, also considered to be components of the central circadian oscillator, are *CCA1* and *LHY*. *LHY* was identified in a screen for mutants displaying elongated hypocotyls and was also shown to be insensitive to photoperiod (flowering at the same time under both long- and short-day conditions). The cloning of *LHY* revealed that it encodes a MYB-related transcription factor with a single MYB repeat, whose mRNA levels oscillate with a 24-h period, peaking each morning (Schaffer *et al.*, 1998). Similarly, *CCA1* is also required for clock function (Green and Tobin, 1999; Wang and Tobin, 1998); is expressed in the morning; and encodes a MYB-related transcription factor with strong sequence similarity to LHY (Wang *et al.*, 1997). Evidence for CCA1 and LHY having integral roles in circadian clock function stems from the fact that in *CCA1* and *LHY* overexpressors, the circadian rhythms of leaf movement, as well as the circadian rhythms associated with *CAB2* expression in entrained plants transferred to continuous light, and *CCR2* expression in continuous darkness, were severely impaired (Schaffer *et al.*, 1998; Wang and Tobin, 1998). Furthermore, *LHY* transcript levels failed to oscillate in plants constitutively overexpressing *CCA1* and, instead, remained at constant, almost undetectable, low levels, indicating that *CCA1* oscillations are required for *LHY* oscillations (Wang and Tobin, 1998). Analysis of *lhy* and *cca1* loss-of-function alleles revealed that *CCA1* and *LHY* have partially overlapping functions: while the single mutants have a shortened-period phenotype for *CAB* expression (Green and Tobin, 1999; Mizoguchi *et al.*, 2002), the double mutants become arrhythmic and flower early under short -day photoperiods (Mizoguchi *et al.*, 2002).

The input pathway leading to the clock involves light signaling from the blue light photoreceptors, the cryptochromes and the red/far-red photoreversible phytochromes. Although the light-input pathway is not fully understood, it is thought to involve intermediates downstream of the photoreceptors such as Early Flowering 3 (ELF3) and the more recently described *TIME FOR COFFEE* (*TIC*) (Hall *et al.*, 2003) that are required to transduce the light signal from the photoreceptors to the central oscillator. Input-pathway components are critical for resetting the clock in response to light, a process often referred to as 'setting the clock to local time'.

The ability of the clock to reset in response to light is required for photoperiod perception (measurement of day length), which is instrumental in timing the transition to flowering. Arabidopsis is a long-day plant, meaning that it flowers earlier in long days (characteristic of the spring) as opposed to short days (characteristic of winter months). Flowering is induced by long-day photoperiods in a clock-dependent manner via the action of *CONSTANS (CO)*, a circadian output gene whose expression peak is late in the evening. CO in turn activates *FLOWERING LOCUS T (FT)* which leads to flowering. However, CO protein is active only in the light, requiring that *CO* expression, and protein accumulation, coincide with the light – a requirement satisfied only under long days (Suarez-Lopez *et al.*, 2001; Valverde *et al.*, 2004). Because the circadian clock is required for the proper timing of flowering, genetic screens for flowering-time mutants have yielded several interesting circadian clock-related genes, including *ELF3* and *ELF4*.

The *ELF3* gene encodes a 695-aa protein with little homology to previously characterized proteins (Hicks *et al.*, 2001). To study the role of *ELF3* in circadian clock function, the *CAB2::LUC* reporter was introduced into an *elf3* mutant background. When *elf3* mutants were entrained to light–dark cycles and transferred to continuous darkness, *CAB2::LUC* levels displayed normal circadian rhythms. However, when transferred to continuous light, *elf3* mutants were arrhythmic, indicating that *ELF3* functions in the light, not in the dark, and is therefore not part of the central oscillator, but more probably part of the light-input pathway (Hicks *et al.*, 1996). A role for *ELF3* in light input was further corroborated by a study placing it within the *zeitnehmer* feedback loop – a somewhat hypothetical input pathway that oscillates in a circadian manner, upstream of the central oscillator, so as to create rhythmic input even under constant conditions (McWatters *et al.*, 2000).

ELF4 has been shown to be required for circadian clock function with respect to both the maintenance of output oscillations (*CAB2* and *CCR2*) and the expression levels of the presumed central oscillator component *CCA1* (Doyle *et al.*, 2002). A requirement for ELF4 in phytochrome-mediated inhibition of hypocotyl elongation has also been demonstrated (Khanna *et al.*, 2003), as well as phytochrome dependence of light induced *ELF4* expression (Khanna *et al.*, 2003; Tepperman *et al.*, 2001). These data suggest an important role for ELF4 in circadian clock function and phytochrome signaling to the clock. Recent reports have indicated that the previously accepted paradigm for the Arabidopsis circadian clock, namely that the central oscillator consists of a single autoregulatory negative-feedback loop, is overly simplified (Farre *et al.*, 2005; Locke *et al.*, 2005), and a theoretical modeling analysis has suggested that ELF4 may be a crucial component of the central oscillator (Locke *et al.*, 2005). Here we provide experimental evidence that ELF4 functions very close to, if not as part of, the central oscillator. More specifically, we present data indicating that ELF4 is a component of a negative-feedback

loop involving the myb-related transcription factors CCA1 and LHY, and acts downstream of the *zeitnehmer* component, ELF3.

Results

Light initiates oscillations in central oscillator gene expression on first exposure of dark-grown seedlings

To identify components of the light-input pathway to the clock, in the absence of the influence of the feedback loops that function under steady-state oscillatory conditions, we have sought to define and utilize the initiation of oscillations in the central clock components by light. Previously we reported that, in seedlings grown in constant darkness from germination onwards, a biphasic waveform of *CCA1* and *LHY* expression is observed during the 24 h after initial exposure to constant red light (Rc) (Kaczorowski and Quail, 2003). To determine whether this waveform is specifically induced by the Rc exposure, and to expand the analysis to include *TOC1*, we monitored the expression of *CCA1*, *LHY* and *TOC1* in seedlings either exposed to Rc or retained in continuous darkness during this 24-h period. As shown in Figure 1(a,b), the expected biphasic oscillations in *CCA1* and *LHY* expression were observed in Rc-exposed seedlings, whereas no detectable deviation in the existing steady-state levels of these transcripts was observed in the dark over this period. As shown in Figure 1(c), *TOC1* expression was also induced by Rc, displaying a broad peak centered at approximately 9 h, counter-phased to the *CCA1/LHY* profiles. Once again, *TOC1* expression remained constant in seedlings retained in darkness. These data are consistent with those expected if the red light-induced expression profiles of *CCA1*, *LHY* and *TOC1* do indeed represent the 'jumpstarting' of the central circadian oscillator as it is currently modeled (Alabadi *et al.*, 2001). Conversely, it may be argued that this pattern could represent light-induced 'synchronization' of pre-existing oscillations in these components, fully operative, but asynchronous between individual cells, organs or seedlings in the population when germinated, such that the net expression levels measured here molecularly in extracts of seedling populations average out as being constant over time.

If the second of the two *CCA1/LHY* expression peaks observed when seedlings are first exposed to Rc truly represent a 'circadian' peak indicative of the initiation of synchronous circadian clock function, we might expect the clock to free-run during prolonged exposure to Rc. To test this, we maintained 4-day-old, dark-grown, wild-type (col) seedlings in Rc for 3 days and examined the expression of *CCA1*, *LHY* and *TOC1* over this 72-h time course. As shown in Figure 2(a,b,d), *CCA1* and *LHY* expression exhibited continued robust oscillations with a 24-h period, peaking at subjective dawn. *TOC1* mRNA levels also oscillated in prolonged Rc, displaying peaks of expression during the subjective night (Figure 2c,d), just as has been reported for plants entrained under light–dark cycles and transferred to

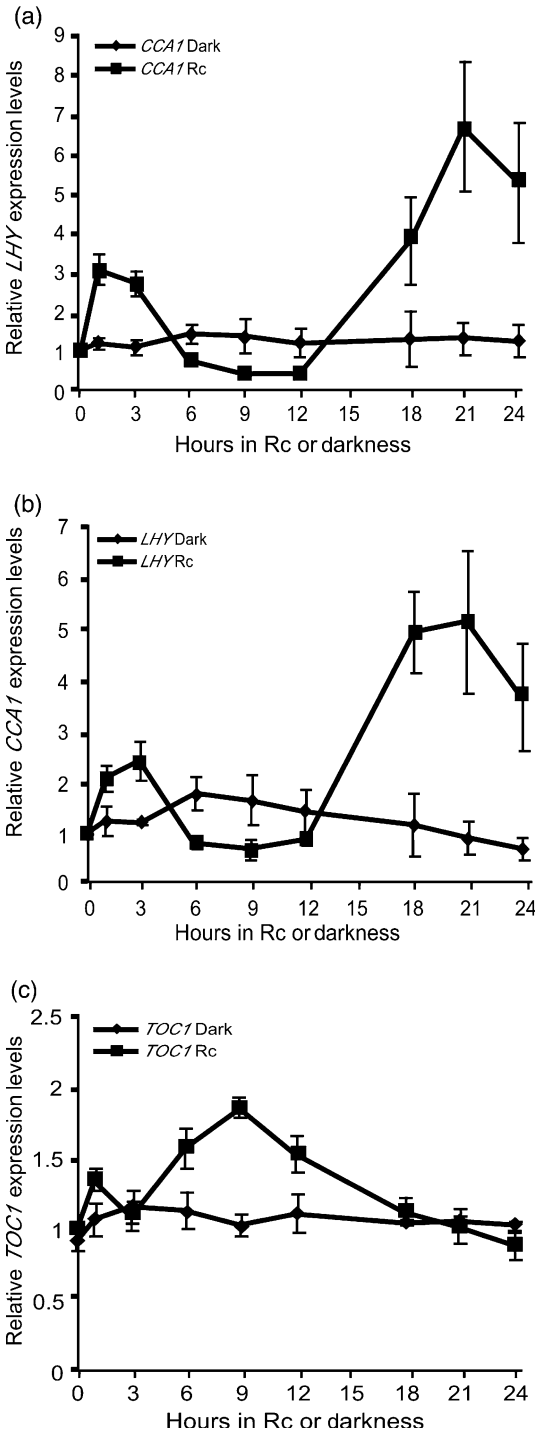

Figure 1. The proposed central oscillator of Arabidopsis is not oscillating in the dark, but is activated in response to light.
Relative (a) *CCA1*; (b) *LHY*; (c) *TOC1* expression levels in 4-day-old, dark-grown, wild-type (col) seedlings either kept in the dark (diamonds) or transferred to constant red light for 24 h (squares).

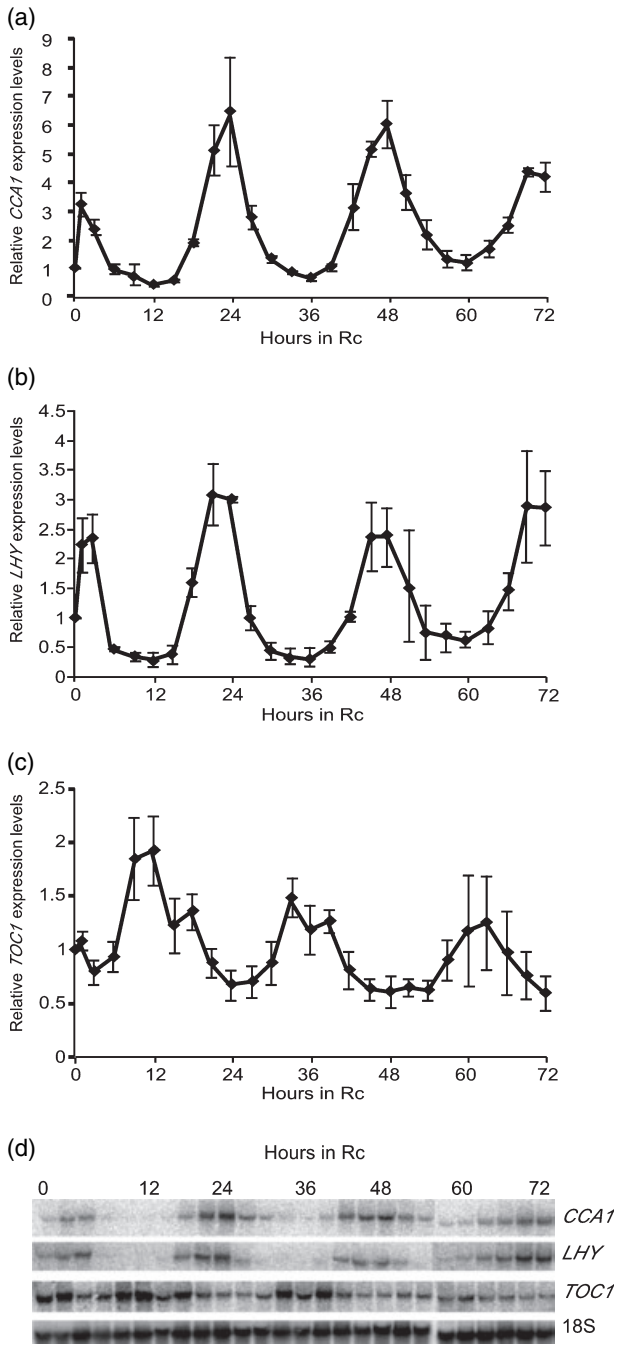

Figure 2. The central oscillator components CCA1, LHY and TOC1 continue to oscillate for at least 3 days in constant red light in the absence of light/dark entrainment.
Relative (a) *CCA1*; (b) *LHY*; (c) *TOC1* expression levels in 4-day-old, dark-grown, wild-type (col) seedlings transferred to constant red light for 72 h; (d) representative Northern blots.

continuous white light (Alabadi *et al.*, 2001). Together with the data indicating that these oscillations are specifically induced by the Rc signal (Figure 1), we conclude that by examining the expression of central oscillator components at the dark-to-red light transition, we can probe the events involved in, and required for, the light-induced initiation of coordinate clock oscillations.

Initial light-induced oscillation in TOC1 *expression occurs independently of CCA1 and LHY*

To examine more closely whether the light-induced, counter-phased, *CCA1/LHY* and *TOC1* profiles observed here are consistent with the model of the central oscillator developed from data derived under free-running oscillatory conditions following light–dark cycle entrainment, we examined *TOC1* expression in seedlings double mutant for *CCA1* and *LHY*. For this purpose we used seedlings that are null for *CCA1*, and also carry a recessive presumptive loss-of-function *lhy* allele that yields a truncated protein. The initial characterization of this double mutant, designated *cca1-1 lhy-12*, was described previously (Mizoguchi *et al.*, 2002). We grew *cca1-1 lhy-12* seedlings in darkness for 4 days and transferred them to Rc for up to 24 h, as above. Interestingly, little effect was observed of the absence of CCA1 and LHY on *TOC1* expression level, Rc-responsiveness or temporal expression pattern (Figure 3). To determine whether the marginal apparent differences between wild type and mutant with respect to *TOC1* expression early in the time course (Figure 3) were statistically significant, a *t*-test was performed for each time point. The 3, 6 and 24-h time points were statistically different between wild-type and mutant with *P*-values of 0.03, 0.03 and 0.01, respectively (Figure 3). At face value, these data are consistent with a quantitatively marginal role for CCA1/LHY in negatively regulating *TOC1* expression, but indicate the absence of an essential functional role of CCA1 and LHY in regulating the overall temporal pattern of *TOC1* expression in etiolated seedlings on initial transfer to Rc. On the other hand, it could be argued that the data represent a small shift in the phase of *TOC1* expression, such that transcript levels peak earlier in the *cca1-1*, *lhy-12* mutant relative to wild type. Nonetheless, even in the absence of CCA1 and LHY, *TOC1* expression is induced on exposure of seedlings to Rc, and downregulated following a peak centered at 6–9 h following first exposure to light. The data therefore suggest that factors acting both positively and negatively on light-induced *TOC1* expression are missing from the current configuration of the central oscillator.

Light-induced oscillations in CCA1 *and* LHY *expression are dependent on TOC1*

Given the somewhat surprising observation that the induction of *TOC1* expression on first exposure to light is only minimally dependent on CCA1 and LHY, we examined the expression of *CCA1/LHY* in a *toc1* mutant. For this purpose, we used *toc1-101*, thought to be a loss-of-function mutant

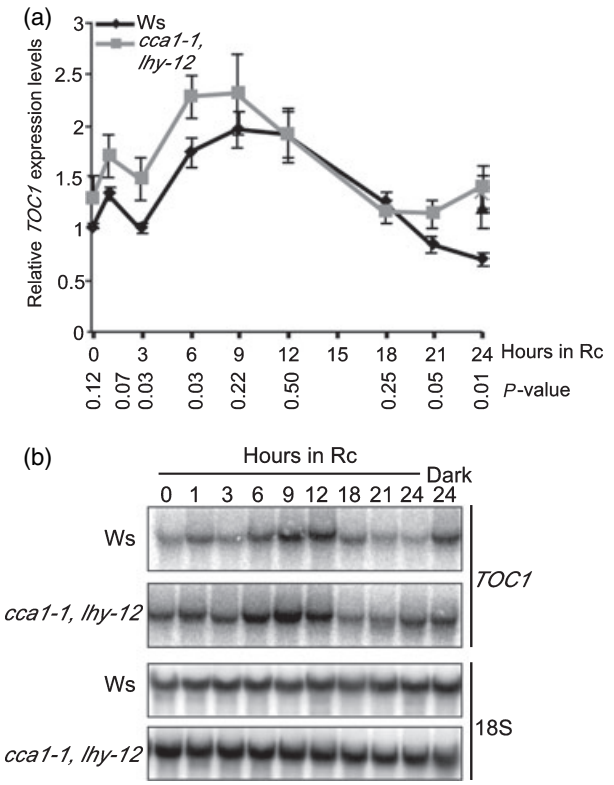

Figure 3. *TOC1* levels are only marginally affected in the *cca1-1 lhy-12* mutant.
(a) Relative *TOC1* expression levels in 4-day-old, dark-grown, wild-type (Ws; black diamonds) and *cca1-1 lhy-12* mutant (gray hexagons) seedlings transferred to constant red light for 24 h. Black triangle and gray cross represent *TOC1* expression levels in wild-type and *cca1-1 lhy-12* seedlings, respectively, maintained in continuous darkness over the time course. *P*-values are results of paired *t*-tests for each time point comparing the wild type with the *cca1-1 lhy-12* mutant.
(b) Representative Northern blot.

due to a frame shift that results in a truncated protein of 188 aa, as described previously (Kaczorowski, 2004). As shown in Figure 4, *CCA1* and *LHY* expression levels were significantly reduced in *toc1-101* compared with wild type, both in the dark and on first exposure to Rc. This result is consistent with TOC1 acting as a general positive regulator of *CCA1/LHY* expression.

However, *CCA1/LHY* expression was not entirely unresponsive to the Rc signal in *toc1-101* seedlings. Instead, both the initial *CCA1/LHY* peak at approximately 1 h and the subsequent peak toward the end of the time-course were still apparent in the mutant, albeit at a significantly reduced amplitude relative to wild type. This result is generally consistent with the role of TOC1 in regulating *CCA1* and *LHY* expression proposed by the central oscillator model. However, the data suggest that, while TOC1 is required for normal levels of *CCA1/LHY* expression, other factors probably function in concert with TOC1 to induce expression at the dark-to-light transition, and to determine the biphasic temporal profile.

ELF4 is required for the red-light induction of CCA1 *and* LHY

To determine whether ELF4 plays a role in light input to the clock, we examined the expression of *CCA1* and *LHY* in the *elf4-101* null mutant under the conditions described above. As shown in Figure 5(a–d), the light-induced expression of both *CCA1* and *LHY* was substantially impaired in *elf4-101* compared with wild-type seedlings under these conditions. Both the acute peaks and the subsequent circadian peaks in the biphasic induction profiles were severely impaired in the *elf4-101* line, much more so than was seen for *toc1-101*. In other words, ELF4 is required both for the initial light-induced expression of these genes, as well as for later light-induced circadian oscillations, underscoring the importance of ELF4 in mediating light input to the central oscillator. These data suggest that the reason *CCA1* expression was reported to be low and arrhythmic in entrained *elf4* plants (Doyle *et al.*, 2002) is because ELF4 is required for the phytochrome-mediated light induction of *CCA1* and *LHY* expression *per se*.

Based on the current central oscillator model involving a negative-feedback loop with CCA1/LHY and TOC1, as well as the overall similarity between *CCA1/LHY* expression in *elf4-101* and *toc1-101* seedlings first exposed to Rc, at least two possibilities for ELF4 function are apparent. The first possibility is that ELF4 could be acting directly on *CCA1/LHY* independently of TOC1. The second possibility is that ELF4 could be acting solely through *TOC1*. To distinguish between these two possibilities, we examined *TOC1* expression in the *elf4-101* mutant. We expected that if ELF4 were acting on *CCA1* and *LHY* independently of TOC1, that *TOC1* expression would be similar to that observed in the *cca1-1 lhy-12* line. If, however, ELF4 were acting on *CCA1* and *LHY* through TOC1, then we expected *TOC1* expression to be reduced in *elf4-101*. As shown in Figure 5(e,f), we saw no effect of the *elf4-101* mutation on *TOC1* expression, consistent with ELF4 functioning independently of TOC1 on *CCA1* and *LHY* expression. Taken together, these data provide evidence that the light-induced expression of *TOC1* is independent of ELF4, and further support the conclusion that factor(s) other than CCA1 and LHY function as a negative regulator of *TOC1* during the declining phase of the time course, and these factor(s) are not dependent on ELF4.

The effect of elf3 *on* CCA1/LHY *expression in Rc is similar to that of* elf4

Based on the data indicating that ELF4 is required for light input to the clock, we hypothesized that ELF4 may function in a way that involves ELF3. If ELF3 and ELF4 were part of the

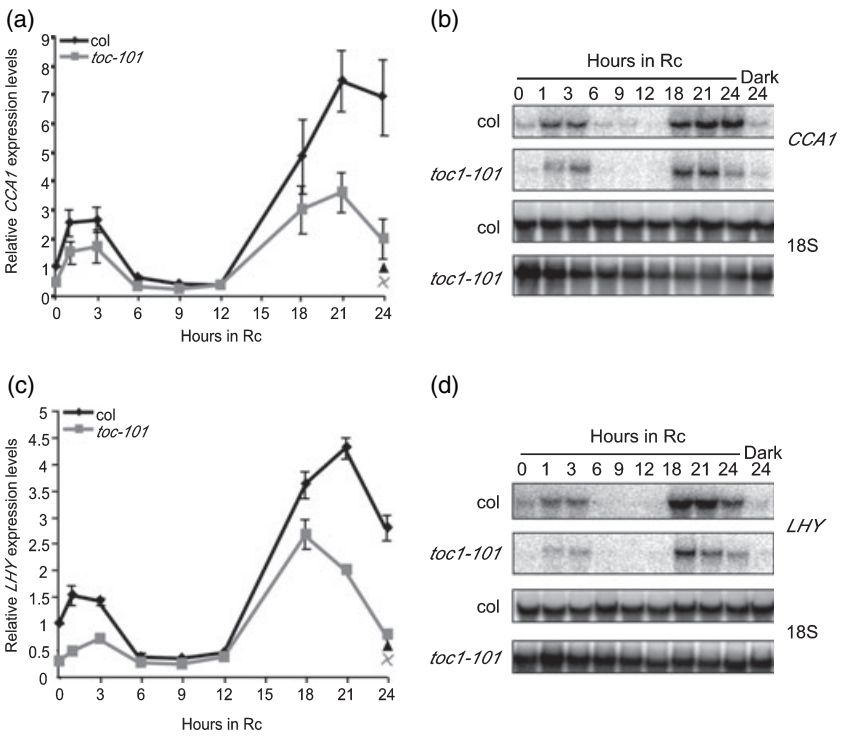

Figure 4. Light-induced *CCA1*/*LHY* expression levels are dependent on TOC1.
Relative (a) *CCA1* and (c) *LHY* expression levels in 4-day-old, dark-grown, wild-type (black diamonds) and *toc1-101* (gray squares) seedlings transferred to constant red light for 24 h. Black triangle and gray cross represent expression levels in wild-type and *toc1-101* seedlings, respectively, maintained in continuous darkness over the time course.
Representative Northern blots: (b) *CCA1*; (d) *LHY*.

same branch of a genetic pathway, then we might expect them to have similar phenotypes. We therefore examined the expression of *CCA1*, *LHY* and *TOC1* in the *elf3-1* mutant in dark-grown seedlings transferred to Rc, as described for *elf4-101* above. The *elf3-1* allele is thought to be a complete loss-of-function allele due to a premature stop codon (Hicks *et al.*, 2001). Not surprisingly, light-induced *CCA1* (Figure 6a,b) and *LHY* (Figure 6c,d) expression was strongly reduced in the *elf3-1* mutant, as was the case for *elf4-101*, except that *LHY* expression was even more reduced in *elf3-1* than in *elf4-101* under these conditions.

Some differences were observed between *elf3-1* and *elf4-101* with respect to *CCA1* and *LHY* expression in the dark, and with respect to *TOC1* expression in the light. Namely, unlike in *elf4-101* (Figure 5e,f), *CCA1* and *LHY* were expressed at lower levels in the dark in *elf3-1* than in wild type (Figure 6a–d). Conversely, while *TOC1* expression in the *elf3-1* mutant was not different to wild type in the dark or for the first 9 h of Rc, this expression remained somewhat elevated relative to wild type during the declining phase of the time course (Figure 6e,f). To determine whether this observed genotype effect is statistically significant, *t*-tests were performed for all data points in the time course to compare the *elf3-1* mutant to wild type. As shown in Figure 6(e), the elevated levels of *TOC1* expression in *elf3-1* are statistically significant at 18 and 24 h Rc ($P = 0.04$ and 0.01, respectively), but not at 12 and 21 h. This possible marginal effect is different from that observed for *elf4-101*, where no effect on *TOC1* levels was observed (Figure 5e).

ELF4 functions downstream of ELF3

Despite the above-mentioned difference, *CCA1*, *LHY* and *TOC1* show highly similar overall expression patterns in *elf3-1* and *elf4-101*, consistent with ELF3 and ELF4 both functioning positively in the same pathway. If this were the case, a simple upstream/downstream relationship could be established by examining the expression of *ELF4* in *elf3-1*, and the expression of *ELF3* in *elf4-101*. The expectation would be that if ELF3 were upstream of ELF4, then *ELF4* expression would be reduced in the *elf3-1* mutant, or *vice versa*. Alternatively, if ELF3 and ELF4 function together as part of a transcriptional negative-feedback loop, we would expect ELF3 to regulate the expression of *ELF4* and ELF4, in turn, to regulate the expression of *ELF3*. Interestingly, as shown in Figure 7(a,b), Rc-induced *ELF4* expression was significantly increased in *elf3-1*. This is clearly inconsistent with the *elf3-1* molecular phenotype being due to down-regulation of *ELF4* in that background. Instead, it suggests that although ELF3 does act upstream of ELF4, it acts as a negative regulator of Rc-induced *ELF4* expression.

To determine whether ELF3 and ELF4 function in a negative-feedback loop, we examined *ELF3* expression in the *elf4-101* mutant. As shown in (Figure 7c,d), there was little or no apparent Rc-induced change in expression, and no significant difference between wild type and *elf4-101* in *ELF3* expression. This result, taken together with the finding that ELF3 negatively regulates Rc-induced *ELF4* expression, is consistent with ELF3 acting upstream of ELF4 without any

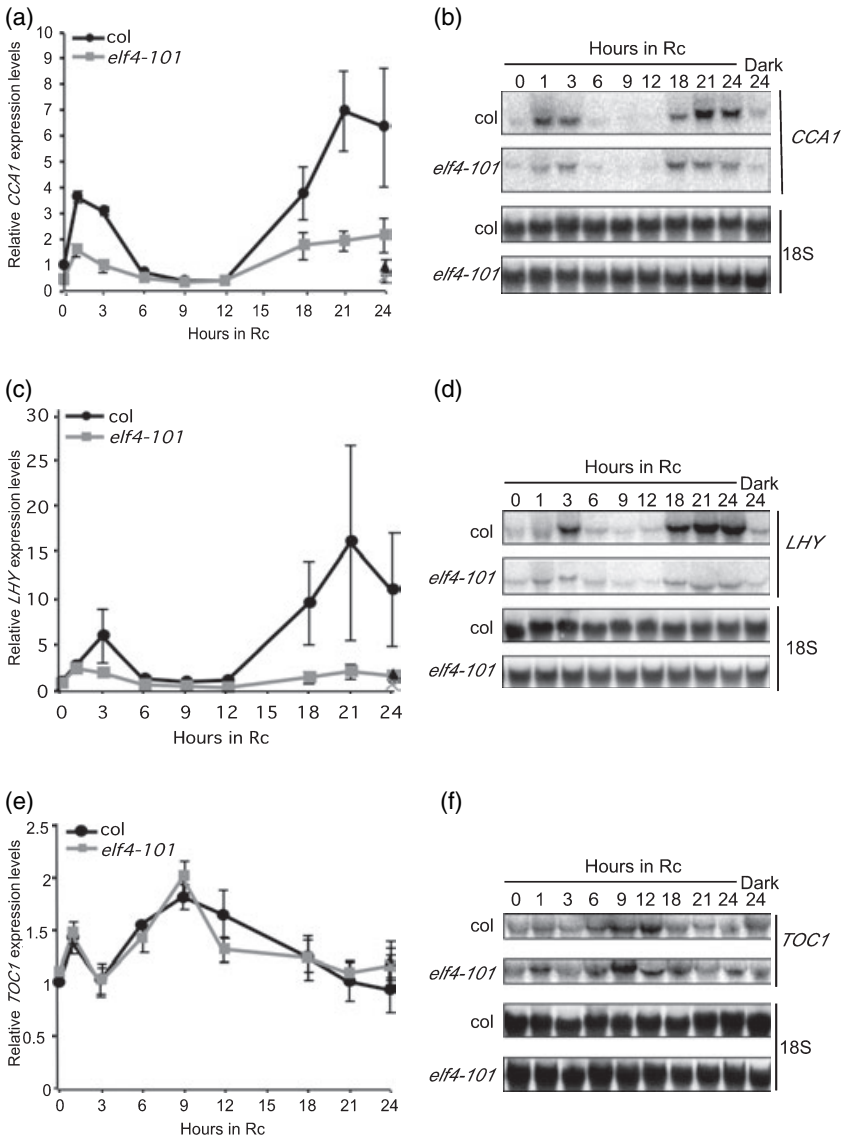

Figure 5. ELF4 is required for the light-induced expression of *CCA1* and *LHY*.
Relative (a) *CCA1*; (c) *LHY*; (e) *TOC1* expression levels in 4-day-old, dark-grown, wild-type (black circles) and *elf4-101* (gray squares) seedlings transferred to constant red light for 24 hours. Black triangle and gray cross represent expression levels in wild-type and *elf4-101* seedlings, respectively, maintained in continuous darkness over the time course.
Representative Northern blots: (b) *CCA1*; (d) *LHY*; (f) *TOC1*.

evidence of a feedback loop. The finding that *ELF3* expression was not significantly light-induced, nor did it appear to oscillate under our conditions, is notable because *ELF3* expression has been shown to oscillate in plants grown in 12 h light:12 h dark cycles as well as under free-running conditions (Hicks *et al.*, 2001). However, a role for ELF3 in regulating *CCA1/LHY* expression at the dark-to-light transition, despite *ELF3* expression itself not responding to the light signal, is consistent with *ELF3* expression, but not *ELF3* oscillations, being required for rhythmic *CCA1* expression in entrained plants (Covington *et al.*, 2001).

CCA1 *and* LHY *negatively regulate light-induced* ELF4 *expression*

The observation that *ELF4* was upregulated more strongly by Rc in the *elf3-1* mutant compared with the wild type raises two alternative possibilities. The first is that ELF3 may act directly on *ELF4* to negatively regulate its light-induced expression. The second is that ELF3 may act indirectly on *ELF4* through another negative factor. The first possibility seemed unlikely because it suggests that *elf3-1* and *elf4-101* would have opposite phenotypes, which they do not. Furthermore, the *ELF4* promoter contains three evening elements that have been shown to be over-represented in genes negatively regulated by CCA1 and LHY (Harmer *et al.*, 2000). Because *CCA1* and *LHY* expression are significantly reduced in the *elf3-1* background, it seemed possible that ELF3 might act indirectly on *ELF4* through CCA1 and LHY. To determine whether this might be the case, we examined *ELF4* mRNA levels in the *cca1-1 lhy-12* double mutant line. As shown in Figure 8(a,b), *ELF4* expression was higher in response to Rc in the *cca1-1 lhy-12* line compared with the wild-type control. To confirm that the differences observed

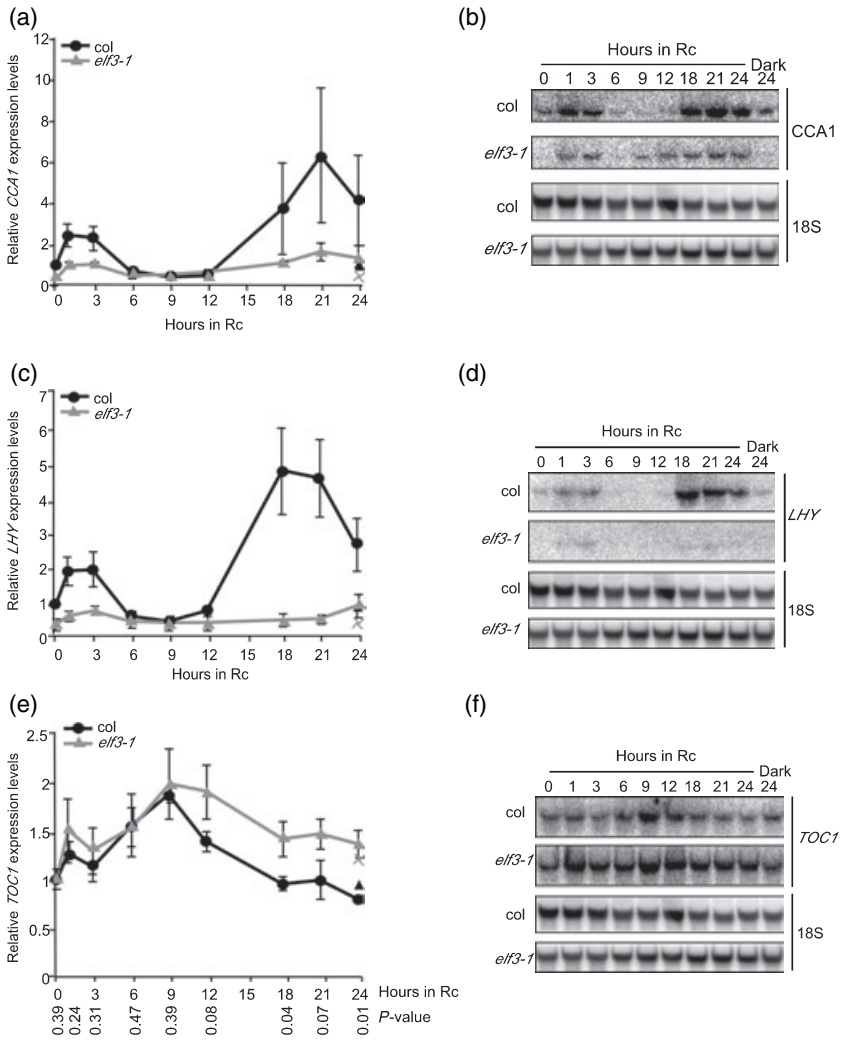

Figure 6. ELF3 is necessary for the light induced expression of *CCA1* and *LHY*.
Relative (a) *CCA1;* (c) *LHY;* (e) *TOC1* expression levels in 4-day-old, dark-grown, wild-type (black circles) and *elf3-1* (gray triangles) seedlings transferred to constant red light for 24 h. Black triangle and gray cross represent expression levels in wild-type and *elf3-101* seedlings, respectively, maintained in continuous darkness over the time course. *P*-values are results of paired *t*-tests for each time point comparing the wild type with the *elf3-1* mutant.
Representative Northern blots: (b) *CCA1;* (d) *LHY;* (f) *TOC1*.

in *ELF4* expression between wild type and mutant were statistically significant, *t*-tests at each data point were performed (Figure 8a). The 3-, 6-, 9-, 21- and 24-h time points showed statistically elevated *ELF4* mRNA levels in the mutant compared with wild type, with *P*-values of 0.01, 0.01, 0.04, 0.02 and 0.002, respectively. However, the overall time course suggests that the main *ELF4* expression peak may occur earlier in the mutant, at approximately 6 h, compared with the wild type where it is centered at approximately 9 h, suggesting that CCA1/LHY may play a predominant role in maintaining the phase or period of *ELF4* expression by controlling the amplitude of oscillation in a temporally specific fashion. Overall, these data indicate that CCA1/LHY negatively regulate *ELF4* in a manner reminiscent of the role described for CCA1/LHY in negatively regulating *TOC1* in the central oscillator model (Alabadi *et al.*, 2001). Consistent with *TOC1* and *ELF4* expression being regulated via similar mechanisms, their mRNA expression in wild-type seedlings follows parallel profiles, with both transcripts having a brief initial minor peak 1 h after dark-grown seedlings are first exposed to Rc, and a broader, second major peak at approximately 9 h (Figures 5e and 7a).

Discussion

Although initially focused on defining the genetic framework for ELF4 function, this study has provided insights into broader questions of light input to, and components comprising, the central circadian oscillator of Arabidopsis. These findings are summarized schematically in Figure 9.

Light-induced initiation of oscillations in net expression of central oscillator components

To investigate the mechanism of light input to the central oscillator and, specifically, the role of ELF4 in this process, we established conditions under which we could monitor the initiation of changes in expression of the proposed central

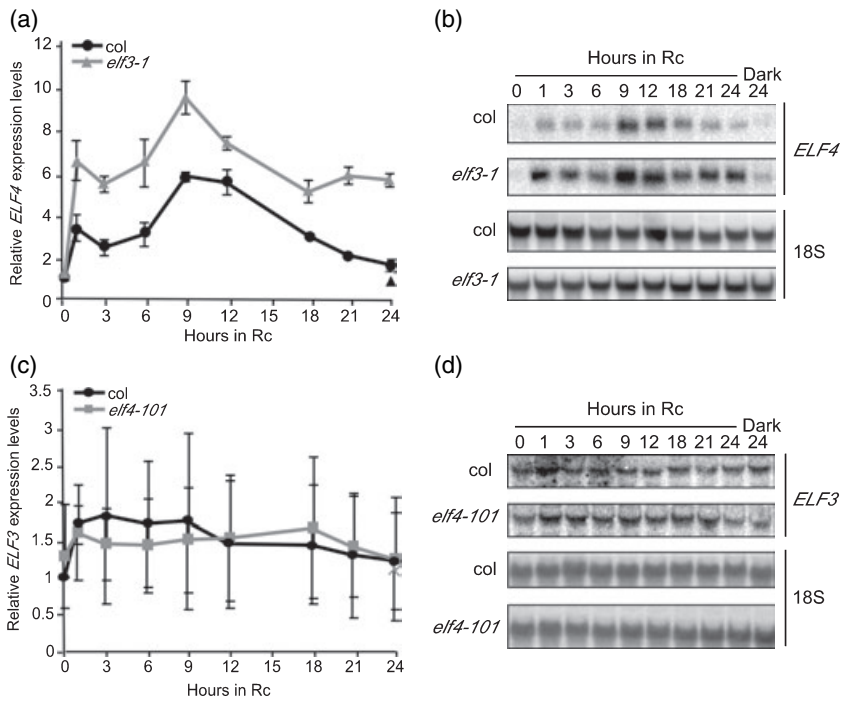

Figure 7. ELF4 functions downstream of ELF3.
Relative (a) *ELF4*; (c) *ELF3* expression levels in 4-day-old, dark-grown, wild-type (black circles) and *elf3-1* (gray triangles) seedlings transferred to constant red light for 24 h. Black triangle and gray cross represent expression levels in wild-type and *elf3-101* seedlings, respectively, maintained in continuous darkness over the time course.
Representative Northern blots: (b) *ELF4*; (d) *ELF3*.

oscillator components CCA1, LHY and TOC1 in response to red light. In completely dark-grown seedlings, *CCA1*, *LHY* and *TOC1* are expressed only at basal levels, with no detectable oscillatory pattern, but are rapidly induced to oscillate on initial exposure to red light (Figure 1). Constant levels of CAB:LUC, *CAT2* and *CAT3* expression were also observed previously in dark-grown Arabidopsis seedlings (Anderson and Kay, 1995; Millar and Kay, 1996; Millar *et al.*, 1992; Zhong *et al.*, 1994, 1998), although rhythmic expression of an *AtCAB2:Luc* transgene in etiolated tobacco seedlings has been reported, possibly indicating a species-specific difference (Kolar *et al.*, 1998). Regardless, the fact that we do not detect *CCA1*, *LHY* and *TOC1* oscillations in the dark suggests that the central oscillator, as defined by these genes, may be poised in a non-oscillating steady-state configuration in dark-grown Arabidopsis seedlings. Alternatively, all individual cells within the dark-grown seedling might contain oscillators that are oscillating autonomously at maximum amplitude in an asynchronous fashion to generate the net constant steady-state levels of *CCA1*, *LHY* and *TOC1*, transcripts observed here in homogenates of a population of whole seedlings (Figure 1). The imaging of individual mouse fibroblasts carrying the *mPER2::LUC* transgene revealed that circadian clocks of individual fibroblasts were synchronized by an external signal and continued to oscillate over the course of 11 days, but drifted out of phase with each other, resulting in the appearance of arrhythmia when examining a population of cells (Welsh *et al.*, 2004). No analogous experiments have yet been reported in plants, so there is currently no direct evidence for or against the possibility of such asynchronous autonomous clocks in cells of these organisms. On the contrary, it has been suggested that, in Arabidopsis, clocks of individual seedlings are always internally synchronized and that external signals, such as imbibition, serve to synchronize populations of seedlings (Zhong *et al.*, 1998). In that study, the authors were unable to detect *CAT2* output-gene oscillations in 'synchronized' etiolated seedlings, but were able to detect circadian gating of the effectiveness of the initial inductive light signal in increasing *CAT2* mRNA levels, leading them to conclude that the central circadian oscillator was functional and synchronized in etiolated seedlings (Zhong *et al.*, 1998). If *CCA1*, *LHY* and *TOC1* are components of the central oscillator ostensibly detected by Zhong *et al.*, we would have expected to measure such oscillations in *CCA1*, *LHY* and *TOC1* expression in dark-grown seedlings. However, we did not. Reconciliation of these data appears to require the existence of another synchronized oscillator not involving *CCA1*, *LHY* and *TOC1* to account for the data of Zhong *et al.* (1998). We argue, therefore, in favor of the hypothesis that the Rc signal in this study serves to initiate the circadian clock, as defined by the currently characterized proposed central oscillator components *CCA1*, *LHY* and *TOC1*.

Regardless of whether the effect of the Rc signal is to perturb constant or synchronize pre-existing oscillating expression levels, we have shown that the induced oscillations in *CCA1/LHY/TOC1* expression, observed during the first day following light exposure, persist for at least 3 days with minimal dampening (Figure 2). This indicates that the clock is functional and synchronized with an approximately 24-h period following exposure to a constant stimulus,

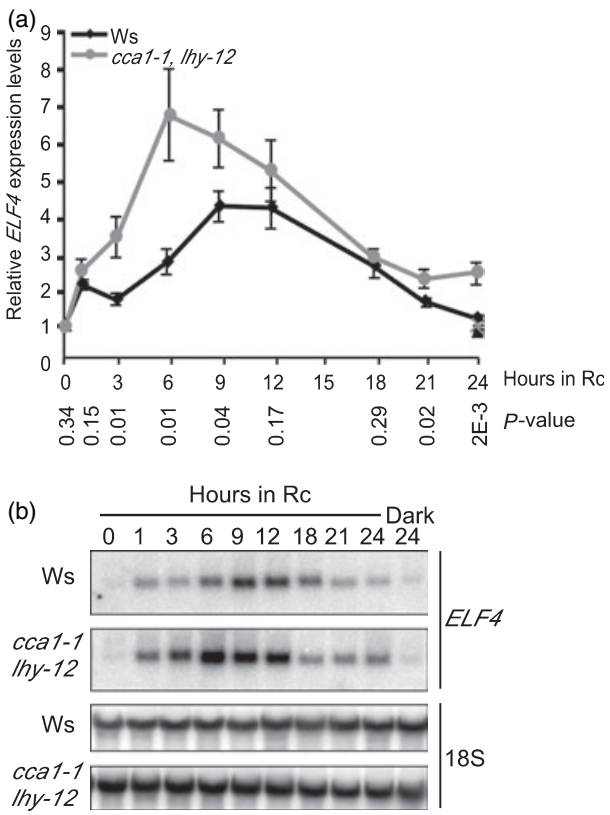

Figure 8. Light-induced *ELF4* expression is enhanced in the *cca1-1 lhy-12* mutant.
(a) Relative *ELF4* expression levels in 4-day-old, dark-grown, wild-type (black diamonds) and *cca1-1 lhy-12* (gray hexagons) seedlings transferred to constant red light for 24 h. Black triangle and gray cross represent *ELF4* expression levels in wild-type and *cca1-1 lhy-12* seedlings, respectively, maintained in continuous darkness over the time course. *P*-values are results of paired *t*-tests for each time point comparing the wild type with the *cca1-1 lhy-12* mutant; (b) representative *ELF4* Northern blot.

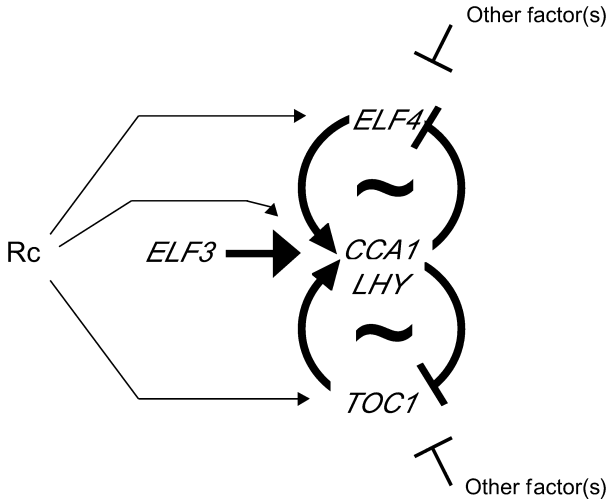

Figure 9. Model showing proposed interlocking feedback loops and multiple points of light regulation of the Arabidopsis circadian oscillator.
Thin arrows indicate points of light input as determined by a change in expression of the indicated gene in response to a red light signal. Heavy lines indicate interlocking transcriptional feedback loops postulated to constitute core elements of the central oscillator. Arrows indicate positive regulation; "T" symbols, negative regulation. The other negative factors acting on *TOC1* and *ELF4* are hypothetical, but are based on the observation that CCA1/LHY activity alone is insufficient to account for the negative regulation of *TOC1* and *ELF4* expression observed over the first 24 h after dark-grown seedlings are first exposed to constant red light.

obviating the need for light–dark entrainment to establish robust oscillations. This is consistent with previous studies showing that output oscillations (*CAB2::LUC*) in untrained, etiolated seedlings treated with a single red-light pulse (Anderson *et al.*, 1997; Somers *et al.*, 1998a) exhibited an initial 'acute' peak of *CAB* expression followed by a second peak at about 24 h following the pulse, similar to those reported here and previously (Kaczorowski and Quail, 2003) for *CCA1/LHY*. However, subsequent *CAB* expression peaks, namely that expected at 48 h, were only marginal in wild-type (L*er*) seedlings, indicative of rapid dampening. The fact that we do not observe strong dampening of *CCA1/LHY* oscillations (Figure 2) may be because our seedlings were kept in Rc rather than continuous darkness following a red-light pulse. Thus our data suggest that the initial waveform pattern observed in Rc-exposed seedlings do indeed represent light-induced initiation of coordinate, repetitive oscillations in these central circadian components, as suggested previously (Kaczorowski and Quail, 2003). Significantly for this conclusion, the data show that *CCA1/LHY* are expressed in antiphase with respect to *TOC1* in dark-grown seedlings first exposed to the light (Figures 1 and 2). This observation is consistent with the antiphased expression of these genes reported for plants entrained to light–dark cycles and transferred to free-running conditions (Alabadi *et al.*, 2001).

CCA1 *and* LHY *expression is dependent on the positively acting factors TOC1, ELF3 and ELF4*

Here we have used the biphasic profiles of net *CCA1/LHY* expression, detectable on first exposure to Rc, as an assay to monitor light input to the clock. By examining the expression of *CCA1* and *LHY* in the monogenic null mutants *toc1-101*, *elf3-1* and *elf4-101*, we have shown that light-induced *CCA1/LHY* expression is reduced in each of these mutants, thereby establishing that all three wild-type alleles at these loci encode positively acting factors that are necessary for normal Rc signaling to the *CCA1/LHY* gene pair (Figure 9). However, the effect of each monogenic mutant on *CCA1/LHY* expression differs to a greater or lesser degree. Namely, *elf3-1* and *elf4-101* most severely impair light-induced *CCA1/LHY* expression, the only notable differences between these two mutants being that *CCA1* and *LHY* were expressed at lower levels in the dark in *elf3-1* than in *elf4-101*, and that *LHY*

levels were more significantly reduced in Rc in the *elf3* mutant than in the *elf4* mutant over the time course.

Despite these relatively subtle differences between *elf3* and *elf4*, the otherwise striking similarity in *CCA1/LHY* expression in these two mutants initially suggested that *ELF3* and *ELF4* may play similar roles in transducing the light signal to the clock. However, the possibility of entirely overlapping functions for *ELF3* and *ELF4* appears to be ruled out because, in the *elf3-1* mutant, *ELF4* is more strongly upregulated in response to Rc than in the wild type. This result suggested a more complex regulation involving other factors and possibly intersection with other genetic pathways. Our data suggest that ELF3 may negatively regulate *ELF4* expression indirectly via the action of CCA1 and LHY (Figure 9).

In contrast to *elf3-1* and *elf4-101* which abolished the initial oscillations in *CCA1/LHY* expression almost entirely, *toc1-101* had a somewhat lesser effect on *CCA1/LHY* levels. This result indicates that, while TOC1 is required for the normal amplitude of Rc-induced *CCA1/LHY* expression, loss of TOC1 can be compensated for, to some extent, by other partially redundant factors, possibly via the action of ELF3 and/or ELF4.

The TOC1 *expression profile is not exclusively dependent on CCA1 and LHY during the first 24 h on exposure to red light*

Similarly to what was observed for *CCA1/LHY* expression in *toc1-101* seedlings, the overall temporal pattern of transient, Rc-induced expression of *TOC1* appears to be only marginally dependent on CCA1 and LHY. The level of *TOC1* expression in the *cca1-1 lhy-12* double mutant appears to be consistently slightly higher than in the wild type, both in the dark and during the early part of the Rc time course (albeit statistically significantly higher only at the 3- and 6-h time points), possibly representing a small shift in the phase of light-induced *TOC1* expression (Figure 3). An earlier phase for *TOC1* expression has been reported previously for *cca1-1 lhy-12* mutant plants entrained to light–dark cycles and transferred to free-running conditions (Mizoguchi *et al.*, 2002). However, no apparent difference in *TOC1* transcript levels is detected between wild type and the *elf4-101* mutant (Figure 5e), which expresses much lower levels of *CCA1* and *LHY* in response to Rc than wild type (Figure 5a–d). Although the *elf3-1* mutant appears to express consistently somewhat elevated levels of *TOC1* over the latter half of the Rc time course relative to wild type (Figure 6e), it is unclear that this results from the strongly reduced levels of *CCA1* and *LHY* expression in this mutant (Figure 6a–d), given the absence of such an effect in the *cca1-1 lhy-12* null mutant (Figure 3). It seems possible, therefore, that only extremely low levels of *CCA1/LHY* transcript, levels as low as those detected in the *elf3* and *elf4* mutants, are required for normal repression of *TOC1* expression.

Existing data derived from plants entrained to light–dark cycles have led to the widely accepted proposal that *CCA1/LHY* and *TOC1* form a feedback loop that probably constitutes the central circadian oscillator, as depicted in Figure 9 (Alabadi *et al.*, 2001). The data presented here are marginally consistent with the operation of such a negative-feedback loop in etiolated seedlings first exposed to Rc, but provide evidence that other factors need to be added to the model to account fully for the observed pattern of *CCA1/LHY/TOC1* gene expression. Specifically, the data presented here suggest the action of a negative factor(s) other than CCA1 or LHY in attenuating *TOC1* expression during the declining phase of the time course (Figure 3), following the initial dark-to-light transition in etiolated seedlings.

ELF4 *is part of a negative-feedback loop involving* CCA1 *and* LHY

In contrast to the *cca1-1 lhy-12* double mutant having only a marginal effect on *TOC1* expression under our conditions, the Rc-induced expression of *ELF4* appears to be significantly higher in both the *cca1-1 lhy-12* and *elf3-1* mutants than in the wild type for part or all of this time course (Figures 7 and 8). This result suggests that CCA1 and LHY may indeed be general negative regulators of *ELF4* expression (Figure 9). This is not the first time that such a model has been proposed. Hayama and Coupland (2004) proposed that CCA1/LHY may negatively regulate *ELF4* expression, but direct evidence has, until now, been lacking. Taken together, the positive regulation of light-induced *CCA1/LHY* expression by ELF4, and the negative regulation of light-induced *ELF4* expression by CCA1/LHY, suggest the existence of an interlocking autoregulatory transcriptional feedback loop working in conjunction with, or parallel to, that previously described for CCA1, LHY and TOC1 (Figure 9). The molecular mechanisms by which TOC1 and ELF4 positively regulate *CCA1/LHY* expression remain unknown. Therefore it is not possible at this point to distinguish whether ELF4 and TOC1 act independently of one another, or function cooperatively, perhaps in a multiprotein complex.

An additional negative-feedback loop involving the pseudo-response regulators PRR7 and PRR9 was proposed recently (Farre *et al.*, 2005). In that study, it was shown under steady-state entrained conditions that PRR7 and PRR9 together negatively regulate the expression of *CCA1* and *LHY*, and that CCA1 and LHY positively regulate the expression of *PRR7* and *PRR9* (Farre *et al.*, 2005). Similar results were also presented by Nakamichi *et al.* (2005). This, together with the data presented here, indicates that the Arabidopsis central circadian oscillator consists of a minimum of three interlocking negative-feedback loops. For simplicity, only the TOC1-containing loop and the ELF4 loop described here are shown in Figure 9.

Interlocking feedback loops are not unheard of in circadian clocks of other systems. The well-characterized circadian clocks of animals and *Neurospora* involve interlocking oscillating feedback loops (Francois, 2005; Preitner *et al.*, 2002). In *Drosophila*, the PAS domain-containing bHLH proteins CLOCK and CYCLE positively regulate the expression of *PER* and *TIM* in one feedback loop; and the basic leucine zipper-encoding gene *VRILLE* in another feedback loop. Completing the loops, PER, TIM and VRILLE all negatively regulate the expression of *CLOCK* and *CYCLE* (reviewed by Van Gelder *et al.*, 2003).

As mentioned above, ELF4 has been implicated in the regulation of the central oscillator in previous studies under entrained conditions (Doyle *et al.*, 2002). However, that study did not directly consider the possibility that ELF4 itself may be a component of a novel negative-feedback loop comprising part of the circadian central oscillator. The fact that loss of ELF4 more severely reduces light-induced *CCA1/LHY* expression than does loss of TOC1, taken together with the fact that loss of CCA1/LHY more severely affects light-induced *ELF4* expression levels than *TOC1* levels, suggests that the ELF4 loop is more active at the initial dark-to-light transition than is the presumptive TOC1-containing loop. This proposition raises the possibility that the TOC1 loop does not become active to the extent described by Alabadi *et al.* (2001) until plants are fully entrained, and transferred to constant conditions for several days, whereas the ELF4 loop, induced and apparently functional at the dark-to-light transition, may act as a light-input loop, required for entrainment, that in turn gives rise to the robust, free-running, antiphased oscillations in *TOC1* and *CCA1/LHY* expression observed by Alabadi *et al.* (2002). Alternatively, the ELF4 loop may be the predominant loop not only under our oscillator induction conditions, but also under entrained steady state, itself generating the output rhythms associated with circadian clock function.

It is notable that because all three factors, ELF3, ELF4 and TOC1, are necessary for the initial rapidly Rc-induced 'acute' peaks of *CCA1* and *LHY* expression (Figures 4–6), the data suggest that these factors all function in the primary phytochrome-signaling pathway that transduces light-input signals to the clock (Figure 9). Moreover, it is also notable that light-induced expression of both *TOC1* and *ELF4* occurs independently of CCA1 and LHY. Together these data provide evidence that there are multiple points of light input to the central oscillator, affecting the oscillator component genes independently (Figure 9).

Experimental procedures

Plant material and growth conditions

Seeds were sterilized in 20% bleach, 0.2% sodium dodecyl sulphate and plated on growth medium plates without sucrose (Hoecker *et al.*, 1999), stratified for 3 days at 4°C in the dark and then exposed to white light for 3 h at room temperature to synchronize germination. Seedlings were then grown for 96 h in the dark at 21°C before being transferred to 7 μmol m^{-2} s^{-1} Rc. Fluence rates were measured with a spectroradiometer (model LI-1800, Li-Cor, Lincoln, NE, USA).

The *elf4-101* allele used in this study is a null allele generated by T-DNA insertion as described previously (Khanna *et al.*, 2003). The *elf3-1* allele has a single base change that results in a premature stop codon resulting in a 350-aa truncation (Hicks *et al.*, 2001). The *cca1-1* allele is a null allele generated by T-DNA insertion (Green and Tobin, 1999). The *lhy-12* allele was generated by mutagenesis of an *LHY* overexpressing line and has a 19-bp deletion, an 11-aa addition, a point mutation and a premature stop codon (Mizoguchi *et al.*, 2002). The *toc1-101* allele was identified by screening a collection of activation-tagged lines, and was shown to have a 16-bp deletion that results in a frameshift and early termination after 188 aa (Kaczorowski, 2004).

RNA isolation and hybridization

Tissue was harvested in the dark at 0, 1, 3, 6, 9, 12, 18, 21 and 24 h after transfer to the light, unless otherwise indicated, and frozen immediately in liquid nitrogen. RNA was isolated from frozen tissue using the Qiagen Plant RNeasy kit according to the manufacturer's instructions (Qiagen, Valencia, CA, USA). For RNA filter blots, 5 μg RNA was run on 1.2% (w/v) agarose gels containing 0.67% (w/v) formaldehyde and transferred to Magna nylon membranes (Osmonics, Westborough, MA, USA) by capillary action in 20× saline sodium citrate buffer. RNA was fixed to the membranes by with a UV-Crosslinker 1800 (Stragene, La Jolla, CA, USA).

Probes for Northern blots were generated either by random priming in the case of *CCA1*, *LHY*, *TOC1* and *ELF3*, or by *in vitro* transcription in the case of *ELF4*. Random priming was performed in the presence of $\alpha[^{32}P]$-dCTP using the Redi-Prime II kit (Amersham Biosciences, Amersham, UK). The *CCA1* probe template was a 949-bp genomic fragment isolated by PCR corresponding to the last 949 bp before the stop codon. The *LHY* probe template was a 1072-bp genomic fragment corresponding to the last 1072 bp before the stop codon. The *TOC1* probe was a 750-bp genomic fragment spanning from the third to the sixth exon (756 bp after the start codon to 1506 bp after the start codon). The *ELF3* probe was a 1.5-kb genomic fragment spanning from 209 bp upstream of start to 1366 bp downstream of start. Hybridization was performed according to Church and Gilbert (1984).

The *ELF4* riboprobe was labeled in the presence of $\alpha[^{32}P]$-UTP using the Riboprobe Transcription System (Promega, Madison, WI, USA). Hybridization was performed as described previously (Khanna *et al.*, 1999).

Blots were visualized using a phosphorimager (Storm 860, Molecular Dynamics, Sunnyvale, CA, USA) and expression levels were quantified using IMAGEQUANT for Mac ver. 1.2 (Molecular Dynamics). Without stripping, blots were re-probed with 18S as a loading control. Expression levels were normalized to 18S and the resulting value at the zero time point for wild-type seedlings was used as a reference and set to equal 1. At least three independent biological replicates were performed, and mean values for each time point were plotted with standard error.

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